Roles for Metal Ions in the Hydrolysis of Adenosine Triphosphate by the 13S Coupling Factors of Bacterial and Mitochondrial Oxidative Phosphorylation†

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ABSTRACT: The ATPase activity of the 13S coupling factor of oxidative phosphorylation from *Alcaligenes faecalis* was enhanced by both monovalent and divalent cations. Activation by Mg^{2+} involves formation of a readily reversible metal–enzyme complex and not the formation of Mg-ATP. The variety of divalent cations that could stimulate the enzyme was much more restricted when monovalent cations were present in the reaction mixture. For example, Ca^{2+} activated just as well as Mg^{2+} in the absence of K^+ but had no effect at all in the presence of K^+. The magnitude of metal ion activation varied with ATP concentration. The extent of K^+ activation decreased with increasing ATP concentration, while that of Mg^{2+} increased with increasing ATP concentration. At low ATP concentration K^+ activated much more strongly than Mg^{2+}. The enhancement of activity by either K^+ or Mg^{2+} alone involves an increase in either the catalytic constant or the rate of release of products or both, without alteration of the affinity of the enzyme for ATP. However, the mechanisms of action of K^+ and Mg^{2+} do not appear to be identical. This is most clearly illustrated by the effect of citrate, which enhanced the extent of Mg^{2+} activation while simultaneously inhibiting the extent of K^+ activation. While ATPase activity was strongly enhanced by added K^+ and Mg^{2+}, some activity was observed without any added cations. EDTA inhibited this cation-independent activity, in addition to preventing activation by K^+ and Mg^{2+}. The mechanism of EDTA action involves formation of a readily reversible complex with the enzyme, not removal of a bound metal from the enzyme. Other metal-complexing agents which form reversible complexes with the enzyme are azide, citrate, and *o*-phenanthroline. The 13S coupling factor from beef heart mitochondria behaved similarly, showing inhibition of cation-independent ATPase activity by EDTA and activation by both monovalent and divalent cations, the extent of which was dependent on ATP concentration.

Recent efforts of this laboratory have been directed toward the elucidation of the reactions occurring between adenine nucleotides and the 13S coupling factors of oxidative and photosynthetic phosphorylation and especially the transformations occurring within the enzyme–nucleotide complex. An understanding of the binding process and the interconversions of coupling factor-bound nucleotides should lead to a better understanding of the chemical steps involved in the synthesis of ATP. The homogeneous 13S coupling factor isolated from spinach chloroplasts, CF_1,[1] binds 2 mol of ADP and converts some of it into AMP and ATP by means of a transphosphorylation reaction (Roy and Moudrianakis, 1971). Similar findings have been reported concerning the 13S, heat-labile coupling factor (HLF) of oxidative phosphorylation from the Gram-negative bacterium *Alcaligenes faecalis* (Adolfsen and Moudrianakis, 1973). One possible explanation of these results is that the reaction represents the terminal step in the synthesis of ATP in both oxidative and photosynthetic phosphorylation.

In the case of HLF, the transphosphorylation reaction has been highly variable from one preparation of enzyme to the next, although quite constant within each preparation of enzyme. In the present article some of the kinetic properties of the ATPase activity of HLF have been studied. The underlying purpose of these studies was to elucidate basic properties and behavior of the enzyme so that some insight into the reason for the variability observed in studies on transphosphorylation might become evident.

Studies on the ATPase activity of coupling factors have usually employed nucleotide concentrations of 1–10 mM. However, since our previous studies on nucleotide binding were carried out at nucleotide concentrations in the vicinity of 10^{-4} M, most of the studies in this paper were performed at this lower concentration of ATP. As a result, some very interesting properties of the enzyme were discovered which would have escaped notice had the studies been performed exclusively at high ATP concentrations. For example, monovalent cations activated the enzyme more strongly than divalent cations at 10^{-4} M but not at 10^{-3} M ATP. Similar results were found for the mitochondrial coupling factor, F_1.

Another way in which the studies presented here differ from those of others is that they were performed on purified enzyme preparations which had *not* been treated in any way to enhance the rate of ATPase activity. The low level of activity which is catalyzed by the enzyme without any "activation" by heat treatment, trypsinization, or incubation with dithiothreitol will be referred to as "intrinsic" activity. The term "intrinsic" is meant to essentially replace the term "latent," since "latent" implies that the unactivated enzyme has no hydrolytic activity at all. The unactivated enzyme simply has a level of activity low enough to escape detection by ATPase assays which detect P_i released from ATP by colorimetric reactions. We felt that it was important to study

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[1] Abbreviations used are: HLF, heat-labile factor, the 13S coupling factor from *Alcaligenes faecalis*; F_1, the 13S coupling factor from beef heart mitochondria; CF_1, the 13S coupling factor from spinach chloroplasts.

the unactivated, intrinsic level of activity because of the possibility that the rather harsh treatments involved in activating the enzyme might seriously alter basic catalytic properties of the enzyme and give rise to kinetic artifacts. Studies on the intrinsic level of activity may yield information more specifically pertinent to the chemical mechanism of ATP synthesis. Also, since these studies were carried out mostly at low ATP concentration and on the intrinsic level of hydrolytic activity, they may be more readily applicable to the *in vivo* function of the enzyme than those performed on activated enzymes under saturating ATP conditions.

Methods and Materials

HLF was purified by a modification of the previously described method (Adolfsen and Moudrianakis, 1971), which employed stepwise elution from DEAE-Sephadex using phosphate buffer instead of elution in a continuous gradient of Tris buffer. This method, which is described in detail elsewhere (Adolfsen and Moudrianakis, manuscript in preparation), consistently gave higher yields of enzyme than the original method. F_1 was purified to homogeneity or near-homogeneity from the high-speed supernatant fraction of mitochondria French-pressed in 0.25 M sucrose by the same method.

ATPase activity was assayed in a reaction mixture containing 50 or 100 μM ATP, 50 μM $MgCl_2$, 30 mM KCl, 5–10 mM Tris or Tricine (pH 8.5), and about 30,000 cpm of [γ-^{32}P]ATP. Reagent [γ-^{32}P]ATP was prepared as previously described (Adolfsen and Moudrianakis, 1973). HLF or F_1 was diluted to a concentration of 20 μg/ml in 10 mM Tris or Tricine (pH 8.5), and the reaction was initiated by the addition of 0.05 ml of enzyme (1 μg) to 0.20 ml of reaction mixture. The system was incubated at 37° for 10 min in the case of HLF or 2 min in the case of F_1. The incubation was terminated by the addition of 0.25 ml of 10% perchloric acid, the systems were chilled on ice, and 0.5 ml of 5% perchloric acid, containing 4 mg/ml of Norit A, was added. Charcoal and adsorbed nucleotide were removed by centrifugation, and 0.5 ml of the supernatant fraction was plated, dried, and counted in a Nuclear-Chicago gas-flow counter. Alternatively, 0.5 ml of the supernatant fraction was counted with 5 ml of water in a Packard Model 2002 liquid scintillation spectrometer. A reagent blank was treated in a manner identical with that of the experimental systems, except that enzyme was replaced by 0.05 ml of buffer.

The enzyme was used as soon as possible after dilution because a slow loss of activity occurred as a result of incubation at 0°. In the case of HLF, time courses of the reaction were linear within experimental error for up to 20 min, and this amounted to about 20% hydrolysis of substrate. Normally the determination of initial velocity involved 5–10% hydrolysis of substrate. The pH optimum was about 8.5. Linearity was observed in the relationship between enzyme concentration and ATP hydrolysis from 1–60 μg of enzyme. The reaction mixture usually contained 1 μg of HLF (4 μg/ml).

Results

Activation of ATPase by Monovalent Cations. The effect of KCl on intrinsic ATPase activity is shown in Figure 1A; the initial stimulation was followed by inhibition at higher concentrations. Since studies on nucleotide binding showed considerable variation from one preparation of enzyme to the next (Adolfsen and Moudrianakis, 1973), many of the studies

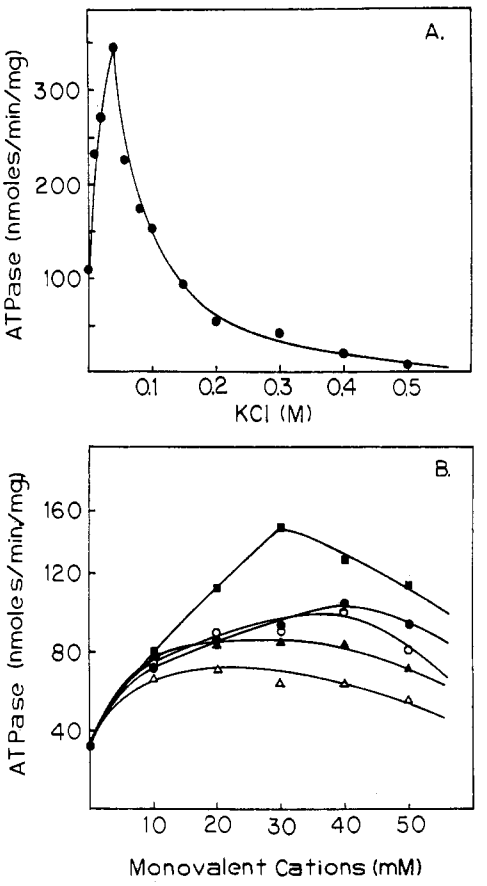

FIGURE 1: (A) Effect of KCl on ATPase activity of HLF. Activity was assayed as described under Methods in a reaction mixture containing 50 μM ATP and the indicated amount of KCl. (B) Activation curves for various monovalent cations: (Δ) NH_4Cl; (▲) LiCl; (○) NaCl; (●) KCl; (■) Tris-Cl.

reported in this paper were done on more than one preparation of enzyme. The stimulation by KCl was about 3.5-fold in the experiment shown in Figure 1. It has varied from two- to tenfold from one preparation of enzyme to the next. Most of this variation was due to differences in the level of intrinsic ATPase activity that was observable in the absence of added monovalent or divalent cations. The specific activity in the presence of 30 mM KCl varied between 150 and 350 nmol/min per mg. Activation by monovalent cations was also found in preparations of beef heart mitochondrial coupling factor, F_1, with 30 mM KCl giving a two- to threefold enhancement of activity.

To test whether the activation was dependent on a specific cation, a specific anion, or simply on the total ionic strength of the medium, a number of different chloride salts of monovalent cations were tested. The activation curves should be exactly superimposable if the activation were due to chloride concentration or total ionic strength of the medium. The data in Figure 1B suggest that the most important event is a rather nonspecific, cation-dependent activation. It is of interest to note that the best activator was the organic cation Tris.

Activation of ATPase by Divalent Cations. It is noteworthy that the HLF preparations did not require the addition of any divalent cations to express intrinsic hydrolytic activity. This was also true for the preparations of F_1. A comparison of the stimulatory effect of various divalent cations on these two enzymes is shown in Table I. In the case of HLF, Mg^{2+}, Ca^{2+}, and Cd^{2+} were stimulatory at 10^{-4} M, while Mn^{2+} was not.

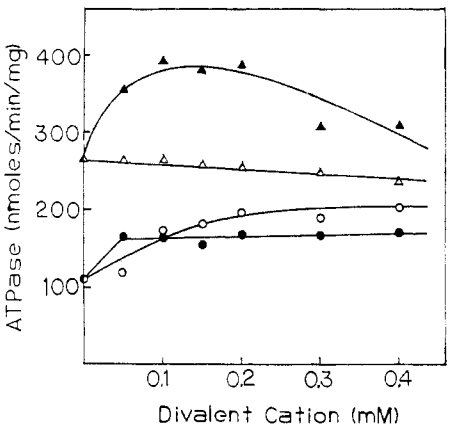

FIGURE 2: Effect of Mg^{2+} and Ca^{2+} on ATPase activity of HLF in the presence or absence of K^+. The reaction mixture contained 50 μM ATP and the indicated concentrations of Mg^{2+} or Ca^{2+} with or without 40 mM K^+: (●) Mg^{2+}; (○) Ca^{2+}; (▲) Mg^{2+}, K^+; (△) Ca^{2+}, K^+.

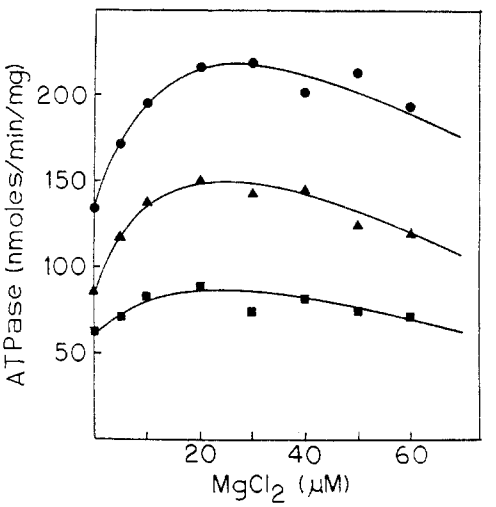

FIGURE 3: Mechanism of activation of ATPase of HLF by Mg^{2+}. The reaction mixture contained the indicated concentration of Mg^{2+} and 12 μM ATP (■), 24 μM ATP (▲), or 48 μM ATP (●).

In the case of F_1 a greater selectivity was observed, with only Mg^{2+} being stimulatory. The maximal extent of activation was usually about 1.5-fold in preparations of either HLF or F_1. This may be compared to the effect of monovalent cations, which gave up to a tenfold stimulation of HLF ATPase and a twofold stimulation of F_1 ATPase. The effect of the metal chelator, EDTA, was of great interest. The results were identical for both HLF and F_1, with 50% inhibition occurring at 10^{-4} M and 97% inhibition occurring at 10^{-3} M. Interaction of EDTA with HLF will be dealt with in greater detail below.

The selectivity of the activation of HLF by divalent cations was markedly different in the presence of K^+. The data in Figure 2 show that both Ca^{2+} and Mg^{2+} activated the enzyme in the *absence* of K^+, and that Ca^{2+} was actually slightly better than Mg^{2+} at the higher concentrations. In the *presence* of K^+, however, Ca^{2+} was not an activator at all. The extent of this increased selectivity for divalent cations became evident in tests of the effect of other divalent cations in the presence of 30 mM KCl. With K^+ present Cd^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} did not activate. The only divalent cation that could replace Mg^{2+} when K^+ was present was Fe^{2+}.

TABLE I: Effect of Various Divalent Cations on ATPase of HLF and F_1.[a]

System	ATPase (nmol/min per mg) HLF	F_1
Control	154	325
10^{-4} M $MgCl_2$	199	460
10^{-3} M $MgCl_2$	161	409
10^{-4} M $CaCl_2$	220	286
10^{-3} M $CaCl_2$	185	263
10^{-4} M $MnCl_2$	141	187
10^{-3} M $MnCl_2$	84	182
10^{-4} M $CdCl_2$	196	291
10^{-3} M $CdCl_2$	144	222
10^{-4} M EDTA	75	164
10^{-3} M EDTA	6	10

[a] The assay system contained 10^{-4} M ATP, 10 mM Tricine (pH 8.5), and additions as noted. The activity was assayed as described under Methods.

The activation by Mg^{2+} could either be due to formation of a metal–enzyme complex or due to formation of Mg-ATP, which might be a better substrate for hydrolysis than free ATP. To distinguish which mechanism was operative in this case, Mg^{2+} concentration was varied at several constant ATP concentrations. If the activation were due to formation of Mg-ATP, then Mg^{2+} should have increased the ATPase activity up to a point corresponding to complete conversion of ATP to Mg-ATP. If it were due to free metal ion activation, the optimum concentration for activation should be independent of ATP concentration. The results in Figure 3 show that optimal ATPase activity occurred at 20–30 μM Mg^{2+} and that it was not affected by a fourfold increase in ATP concentration. This suggests that Mg^{2+} enhanced the enzymatic activity by forming a metal–enzyme complex.

The rate of interaction of Mg^{2+} with the enzyme was tested by performing a time course of ATP hydrolysis with and without Mg^{2+} in the reaction mixture. If the formation of the metal–enzyme complex were slow, then the time course in the system containing Mg^{2+} should show a lag followed by an acceleration, as the more active metal–enzyme complex was formed. The time course of both the experimental and control systems was linear from 2 to 20 min. Thus, formation of the metal–enzyme complex appeared to be relatively rapid, reaching completion in less than 2 min.

The strength of the metal–enzyme complex was assessed in a dilution test. Enzyme was incubated with 10^{-4} M Mg^{2+} for 5 min at room temperature and then diluted prior to assay so that the contribution of Mg^{2+} in the volume of enzyme added to the reaction mixture was only 2 μM. The ATPase assay was conducted with or without 50 μM $MgCl_2$ added to the reaction mixture. If the enzyme–metal complex were stable, then the dilution should *not* result in dissociation to free metal and free enzyme, and the supplementation of the reaction mixture with Mg^{2+} should *not* give any stimulation of ATPase activity. The usual amount of stimulation by Mg^{2+} in the reaction mixture was observed. Thus, the metal–enzyme complex does not appear to be stable; free metal and enzyme-bound metal appear to be in rapid equilibrium.

ATP Concentration and Cation Activation. ATP saturation curves in the presence and absence of monovalent and divalent cations are shown in Figure 4A. Double reciprocal plots were linear (not shown) and indicated that the cations affected

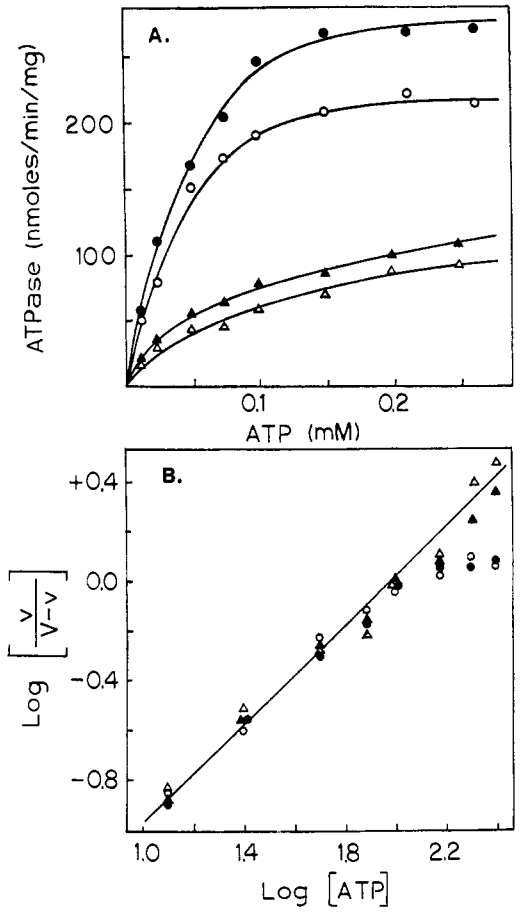

FIGURE 4: Saturation curves (A) and Hill plots (B) for HLF ATPase: (Δ) no cations; (▲) 20 μM Mg^{2+}; (○) 30 mM K^+; (●) 20 μM Mg^{2+}, 30 mM K^+.

V_{max} without affecting K_m. The K_m was 0.10 mM in *all cases*, and the maximal velocities in nanomoles/minute per milligram were 120 in the absence of added cations, 250 in the presence of Mg^{2+}, 400 in the presence of K^+, and 500 in the presence of both Mg^{2+} and K^+. The double reciprocal plots for the systems containing K^+ showed a *slight deviation* from linearity at the higher concentrations of ATP. Also, the observed maximal velocities of the systems containing K^+ (Figure 4A) were only about half of the maximal velocities estimated from the double reciprocal plots. Figure 4B shows a Hill plot of these data, with a line of slope 1.0 drawn through the experimental points. All systems obeyed a linear relationship at low ATP concentrations, while a significant deviation occurred at higher concentrations when K^+ was present.

The data in Figure 4A also show that the magnitude of K^+ stimulation varied with ATP concentration. In another saturation curve extending up to 0.8 mM ATP, the activity of the system without any added cations continued to increase, while the activity of the system containing K^+ did not. Also, the curves for the systems with and without Mg^{2+} diverged, with the activity of the system containing Mg^{2+} increasing at a slightly faster rate than the system without Mg^{2+}. The net result of these changes is summed up in Table II, in which the cation stimulation patterns were determined for both HLF and F_1 at a concentration of 1 mM ATP. For HLF, the stimulation by Mg^{2+} was about twofold, compared to 1.5-fold at low ATP concentration, and the stimulation by K^+ was about twofold, compared to fivefold at low ATP concentration. In the case of F_1, the stimulation by Mg^{2+} averaged 3.6-fold, compared to about 1.5-fold at low ATP concentration, while stimulation by K^+ averaged 1.3-fold, compared to about twofold at low ATP concentration. *In summary, K^+ gives greater stimulation at low ATP concentration, while Mg^{2+} gives greater stimulation at high ATP concentration.*

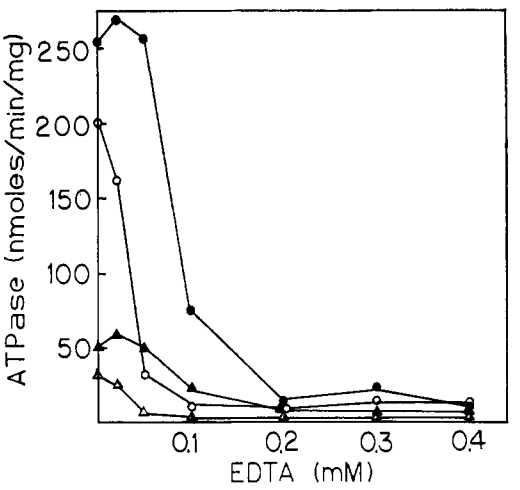

FIGURE 5: Effect of EDTA on ATPase of HLF. The reaction mixture contained 50 μM ATP–EDTA at the indicated concentrations and either no cations (Δ), 50 μM Mg^{2+} (▲), 30 mM K^+ (○), or 50 μM Mg^{2+}, 30 mM K^+ (●).

Interaction of EDTA with HLF. The concentration dependence of the EDTA effect observed in Table I is shown in Figure 5. In the presence of Mg^{2+} there was a slight stimulation of ATPase activity by 20 μM EDTA before the inhibition began. In some preparations of enzyme this stimulation was also seen in the presence of K^+. It is of interest to note that inhibition by EDTA was never complete; maximal inhibition was about 95% in all four cases.

The fact that EDTA is a chelator of divalent cations and that it inhibits the ATPase activity observed in the absence of added cations raises the possibility that the chelator may be interacting with metal ion that is bound very tightly to the enzyme. If this is so, then EDTA could inhibit either by forming an *in situ* complex with the bound metal or by removal of the metal from the enzyme. The mechanism of EDTA

TABLE II: Cation Activation of HLF and F_1 ATPase at High ATP Concentration.[a]

Additions	ATPase (nmol/min per mg) HLF	F_1
None	68	710
10^{-4} M $MgCl_2$	146	3240
30 mM KCl	135	980
10^{-4} M $MgCl_2$ + 30 mM KCl	241	3660

[a] The reaction mixture contained 1 mM ATP, 10 mM Tris (pH 8.5), and additions as noted. Systems with HLF contained 10 μg of enzyme protein and were incubated for 10 min at 37°. Systems with F_1 contained 1 μg of protein and were incubated for 10 min at 37°. To assure complete adsorption of nucleotide after the addition of acid, charcoal was increased from 2 to 10 mg.

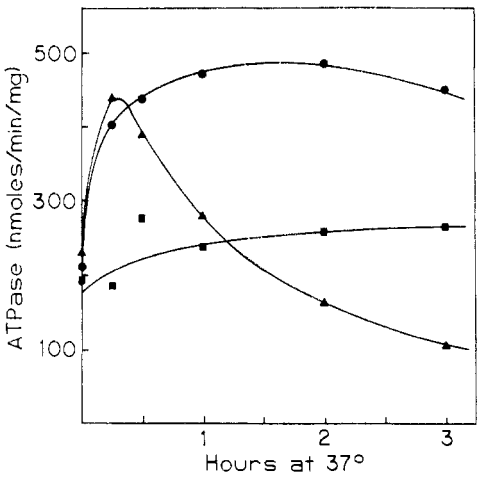

FIGURE 6: Effect of preincubation of HLF in Mg^{2+} or EDTA on heat activation of ATPase activity. HLF was preincubated for the indicated lengths of time at 37° at a concentration of 20 μg/ml in 10 mM Tris (pH 8.5) with either no cations (■), 10^{-4} M Mg^{2+} (●), or 10^{-4} M EDTA (▲). The reaction mixture contained 50 μM ATP and 30 mM KCl.

action was investigated in a dilution test. The enzyme was preincubated in 1 mM EDTA for 10 min at room temperature and then diluted so that the EDTA contribution of the volume of preincubated enzyme to the reaction mixture was 20 μM or less. Enzyme treated in this manner had just as much hydrolytic activity as the control enzyme, which was handled in the same manner but without EDTA present. The apparent reversibility of EDTA inhibition suggested that EDTA was inhibiting *in situ* and not by removal of bound metal.

One possibility not taken into account in the above experiment was that perhaps ATP was required for EDTA to interact with the enzyme. The effect of ATP was assessed by preincubating HLF with EDTA in the presence or absence of ATP (see Table III). In both cases the activity reappeared after dilution of the EDTA to a concentration low enough not to interfere with the initial velocity determination. In fact the activity actually *increased* slightly with respect to the control systems, which were treated in exactly the same manner but without EDTA present. To be sure that EDTA had inhibited the enzyme during the preincubation, a control was run in which the ATP was labeled with [γ-^{32}P]ATP. This system was acidified at the end of the 10-min preincubation period and the amount of ATP hydrolyzed was determined. The specific activity of 0.8 nmol/min per mg (Table III) clearly shows that the hydrolytic activity was inhibited during the preincubation and indicates that it returned upon dilution.

TABLE III: *In Situ* Inhibition of ATPase Activity by EDTA.[a]

Additions to Preincubation System	ATPase (nmol/min per mg)
1. None	138
2. EDTA	167
3. EDTA + ATP	175
4. EDTA + ATP (control)	0.8

[a] Ten microliters of HLF at 1.80 mg/ml was mixed with 80 μl of 10 mM Tris (pH 8.5) + 20 mM KCl, containing (1) no additions, (2) 1 mM EDTA, (3) 1 mM EDTA + 50 μM ATP, and (4) 1 mM EDTA + 50 μM ATP + [γ-^{32}P]ATP. After a 10-min preincubation at 37°, 0.8 ml of 10 mM Tris (pH 8.5) was added and 0.05 ml (1 μg) was taken for assay in a reaction mixture containing 50 μM ATP as described under Methods. The fourth system (control) was acidified without dilution and hydrolyzed ATP was determined by charcoal adsorption as described under Methods.

Activation of HLF ATPase by Heat Treatment. Incubation of HLF at 20 μg/ml in 10 mM Tris (pH 8.5) at 37° resulted in an increase in hydrolytic activity by about threefold over a period of 3 hr. This activation appeared to be unrelated to the activation by K^+ or Mg^{2+} because the heat-activated enzyme was stimulated to the same extent as the unactivated enzyme by these cations. Preparations of HLF stored for several weeks at −20° did not undergo significant heat activation. However, when the enzyme was incubated in the presence of 10^{-4} M Mg^{2+}, heat activation could be demonstrated once again (Figure 6). Mg^{2+} had no significant effect on the heat activation of a preparation of fresh enzyme. The restoration of the ability of the enzyme to heat activate by Mg^{2+} is further evidence for a metal–enzyme complex. EDTA enhanced the ability of preparations of fresh enzyme to heat activate and also restored the ability of aged enzyme preparations to heat activate (Figure 6). This is further evidence for the formation of an HLF–EDTA complex. The decline of activity, which began at about 15 min of incubation, was not observed in all preparations of enzyme.

Effect of Other Metal-Complexing Agents on ATPase Activity. A number of other substances which complex with metal ions also influence ATPase activity. Azide (Figure 7A) inhibits activation by both monovalent and divalent cations, without having any apparent effect on the level of activity observed in the absence of added cations. It is notable that, while azide completely prevented activation by Mg^{2+} and the combination of Mg^{2+} and K^+, the activation by K^+ alone was only partially prevented. Citrate (Figure 7B) completely prevented K^+ activation while *enhancing* activation by Mg^{2+}. It had no effect on cation-independent ATPase activity. On the chance that these effects were due to citrate being a carboxylic acid, the effects of two other carboxylic acids were compared to that of citrate. Sodium acetate (20 mM Na^+, 20 mM acetate), sodium succinate (20 mM Na^+, 10 mM succinate), and sodium citrate (20 mM Na^+, 5 mM citrate) gave specific activities of 102, 165, and 25 nmol/min per mg, while the control of 20 mM NaCl gave a specific activity of 146. Thus, activation by Na^+ was only interfered with significantly by citrate. Another chelator, *o*-phenanthroline, did not inhibit ATPase activity but instead stimulated it in the presence or absence of cations (Figure 7C). The stimulation was significant only at moderately high concentrations (1–2 mM). Hydroxyquinoline (data not shown) had no effect on K^+ activation and only slightly inhibited Mg^{2+} activation at concentrations up to 1 mM. It had no effect on cation-independent ATPase activity.

Dilution tests were performed to assess whether the effects of azide, citrate, and *o*-phenanthroline were permanent or readily reversible. In all cases dilution resulted in a return of the activity to what it was without the substance present. Thus, as in the case with EDTA, these substances appear to form readily reversible complexes with the enzyme. Azide, citrate, and *o*-phenanthroline also enhanced the extent of heat activation in aged preparations of enzyme.

Discussion

Activation by monovalent cations has been observed for

many different enzymes (Suelter, 1970). Perhaps the most widely held opinion as to the mechanism of action is that the monovalent cation binds to the enzyme, and that this induces some sort of change in the enzyme, perhaps conformational, which results in an increase in the catalytic activity. This mechanism was proposed by Kachmar and Boyer (1953) to explain the activation of pyruvate kinase by K^+. Recently another possible mechanism of action of K^+ in pyruvate kinase was proposed by Kayne and Reuben (1970), based on data from nuclear magnetic resonance spectroscopy. A K^+ was found at the active site within 8 Å of a bound Mn^{2+}. In the nuclear magnetic resonance studies of Nowak and Mildvan (1972), this bound K^+ was shown to function by coordinating the carboxyl group of phosphoenolpyruvate at the active site. With respect to the possible role of K^+ for ATPases, Lowenstein (1960) has shown that K^+ can form a ternary complex with Mn-ATP, and that this stimulates the rate of ATP hydrolysis in a model system.

Whether the activation of the ATPase of the 13S coupling factor proceeds by binding of K^+ at the active site or elsewhere on the enzyme cannot be concluded at the present. The lack of cation specificity and the ability of Tris to replace inorganic monovalent cations (Figure 1B) seems to favor action through a less specific mechanism than binding directly at the active site. It should be noted, however, that while no significant degree of specificity was observed in the isolated, homogeneous enzyme, it is quite possible that greater specificity exists when the enzyme is membrane bound.

Na^+ and K^+ have been reported to activate other bacterial 13S ATPases only slightly or not at all (Harold, 1972). Pullman *et al.* (1960) reported no stimulation of F_1 ATPase by KCl or KF. The reason for the lack of stimulation in these cases may be due to the use of high concentrations of ATP in the reaction mixture (Table II) and also high concentrations of buffer, which can replace inorganic monovalent cations (Figure 1B).

Activation by Na^+ and K^+ inevitably yields to speculation as to whether the enzyme is involved in the active transport of these cations. Harold *et al.* (1969) have shown that K^+ transport in *Streptococcus faecalis* is inhibited by dicyclohexylcarbodiimide, an inhibitor of oxidative phosphorylation in mitochondria. The fact that bacterial 13S ATPases are not sensitive to quabain (Harold, 1972), which is a potent inhibitor of the Na^+, K^+-ATPase of mammalian systems, cannot be taken as evidence against the involvement of these enzymes in bacterial ion transport, since other inhibitors which act strongly in higher systems do not function in bacterial systems. Oligomycin, for example, inhibits oxidative phosphorylation of mitochondria but not bacteria. It is of interest to note that oligomycin not only inhibits oxidative phosphorylation in mitochondria but also inhibits Na^+ and K^+ transport (Whittam *et al.*, 1964).

The mechanism of activation by divalent cations appears to involve the formation of a metal–enzyme complex and not the formation of Mg-ATP, since the concentration at which optimal Mg^{2+} stimulation occurred was independent of ATP concentration (Figure 3). Further evidence for a metal–enzyme complex comes from the restoration by Mg^{2+} of the ability of the enzyme to heat activate at 37° (Figure 6). The formation of the metal–enzyme complex was relatively fast and readily reversible, with metal removable from the enzyme simply by decreasing the concentration of free metal by dilution. The looseness of this complex qualifies it as a "metal–enzyme" complex in the terminology of Vallee (1960), as opposed to a metalloenzyme, in which the metal is bound so tightly that

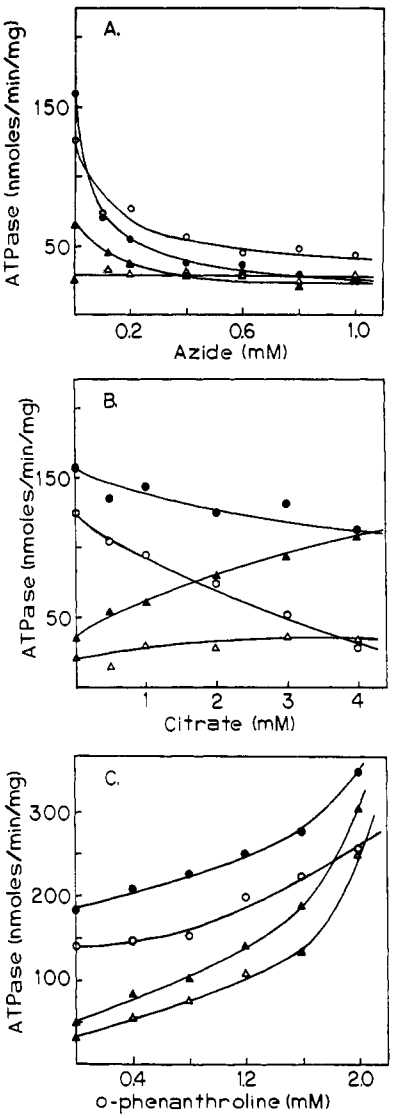

FIGURE 7: Effect of azide, citrate, and *o*-phenanthroline on ATPase activity of HLF. The reaction mixture contained 50 μM ATP, the indicated concentration of azide, citrate, or *o*-phenanthroline, and either no cations (Δ), 50 μM Mg^{2+} (▲), 30 mM K^+ (○), or 50 μm Mg^{2+}, 30 mM K^+ (●).

it is retained through the entire purification procedure. In the case of metal–enzyme complexes it is sometimes difficult to establish what the "native" metal is, since other metals may be similar enough to mimic its effects. In the case of HLF, a number of metals could function in activation (Table I). K^+ increased the specificity of the enzyme for the divalent cation and narrowed the choice of the natural activator down to Mg^{2+} or Fe^{2+}. The former may be favored on the basis of its widespread requirement in enzymes catalyzing reactions involving ATP.

The mechanisms of action of both K^+ and Mg^{2+} involve modification of the maximal velocity of the reaction, not the affinity of the enzyme for nucleotide, since the cations changed the V_{max} but not the K_m. They may exert their influence either by increasing the rate of release of products from the enzyme or by increasing the catalytic constant or both. The opposite responses of the K^+- and Mg^{2+}-enhanced ATPases to citrate (Figure 7B) suggest that the mechanisms of activation, though similar, may actually be independent of each other. This interpretation is consistent with the ability of monovalent

cations to modify the divalent cation specificity (Figure 2). It is also consistent with the opposite responses in the extent of K^+ and Mg^{2+} activation to increasing ATP concentration (Figure 4A).

An interaction coefficient of 1.0 at low ATP concentration in the Hill plot of Figure 4B suggests that hydrolysis involves a single class of noninteracting sites. At higher concentrations, however, negative cooperativity is observed in the systems containing K^+. This implies that there is more than one site on the enzyme with which ATP interacts and that allosteric interactions may occur between these sites. Whether or not these other sites are hydrolytic sites is an open question. An alternative interpretation may be that the apparent negative cooperativity is a manifestation of substrate inhibition, in which a second molecule of ATP may bind at the same hydrolytic site, resulting in a decreased rate of hydrolysis. However, saturation curves of enzymes in which substrate inhibition occurs usually show an optimum, after which activity decreases with further addition of substrate. Since this was *not* observed in Figure 4A, the explanation involving negative cooperativity is favored at the present. Allosteric phenomena have been reported by Nelson *et al.* (1972) in relation to the inhibition of ATPase activity by ADP, and also by Roy and Moudrianakis (1971) in a saturation curve of ADP binding to the coupling factor of photophosphorylation from spinach chloroplasts.

The presence of intrinsic ATPase activity in both HLF and F_1 in the absence of any added divalent or monovalent cations raised the possibility that the enzyme, in addition to being able to form metal–enzyme complexes with Mg^{2+} and K^+, already had a metal bound tightly to it. This was further suggested by the inhibition of this activity by EDTA (Figure 5). The membrane-bound 13S ATPase of *Micrococcus lysodeikticus* has also been reported to have activity in the absence of added divalent cations, which can be inhibited by EDTA (Muñoz *et al.*, 1969). The mechanism of action of EDTA appears to be by formation of an EDTA–enzyme complex and not due to removal of bound metal ions, because dilution of EDTA to a lower concentration resulted in an immediate reversal of the inhibition (Table III). Further evidence for the existence of this complex was that incubation of the enzyme with inhibitory concentrations of EDTA at 37°, followed by dilution of EDTA to a noninhibitory level for the assay, resulted in an *increase* in the maximal extent of heat activation of ATPase (Figure 6). Other metal-complexing agents, azide, citrate, and *o*-phenanthroline, also appear to interact with the enzyme by the formation of readily reversible complexes. Whether or not the site of interaction of metal-complexing agents on the enzyme contains a tightly bound metal is an open question which is currently under active investigation.

One final point in relation to the activation by cations should be made. The 13S coupling factors have been referred to as Mg^{2+}-ATPases or Ca^{2+}-ATPases, with the distinction being made on the basis of which cation gives the *greatest* amount of stimulation. This nomenclature was sufficient at one time but seems to be somewhat confusing now. For example, HLF is a Ca^{2+}-ATPase in the *absence* of monovalent cations, but it is a Mg^{2+}-ATPase in the *presence* of monovalent cations (Figure 2). Considering the effects of monovalent cations, HLF would have to be called a Tris-ATPase, since Tris stimulated activity more than any of the inorganic cations tested (Figure 1B). At high ATP concentrations (1 mM), HLF is a K^+, Mg^{2+}-ATPase, since both cations stimulate to about the same extent (Table II). Also, HLF is a Mg^{2+}-ATPase at low ATP concentration when citrate is present (Figure 7B). The problems in this nomenclature extend to the mitochondrial and chloroplast 13S enzymes also. F_1 is a K^+-ATPase at low ATP concentration but a Mg^{2+}-ATPase at high ATP concentration. In the case of CF_1, Mg^{2+} functioned about half as well as Ca^{2+} in enhancing intrinsic ATPase activity when the assay was performed in Tris–maleate buffer (Karu and Moudrianakis, 1969). Recently, Nelson *et al.* (1972) have studied the effect of maleate on the activated ATPase of CF_1 in much greater depth and have found that Mg^{2+} enhances activity more than Ca^{2+} when the concentration of maleate is 60 mM or greater. Thus, this enzyme can also be operationally referred to as either a Ca^{2+}-ATPase or a Mg^{2+}-ATPase, depending on assay conditions. Since the assay conditions can modify the ionic dependencies of the reaction so drastically, the old nomenclature becomes confusing and actually meaningless. We recommend that it be abandoned and that some other characteristic of the enzyme be used to describe its activity. A physical characteristic such as size in addition to its source or origin appears to us to be appropriate. Under such a nomenclature, HLF, F_1, and CF_1 may be referred to as the 13S ATPases from *A. faecalis*, beef heart, and spinach, respectively.

By way of conclusion we would like to point out that there are two known modes of interaction of nucleotides with HLF. The previously characterized enzyme–nucleotide complex (Adolfsen and Moudrianakis, 1973) was referred to as a "tight" complex because it takes between 1 and 2 hr of incubation at 37° for the formation of the HLF–ADP complex to reach completion, and because once it is formed it is stable enough to be isolated by exclusion chromatography on Sephadex G-50M columns. The enzyme-bound nucleotides can serve as a substrate *from within* the microenvironment of the active-site region of the enzyme for reactions which can be subsequently monitored by isolating the enzyme–nucleotide complex, acid-extracting the enzyme-bound radionucleotide, and then identifying the radionucleotide by a procedure involving charcoal and Dowex columns. It is conceptually very important to understand that the very slow formation and dissociation of the enzyme–nucleotide complex dictates that the products of such reactions will remain mostly enzyme bound. Thus, they bear a stoichiometric relationship to the amount of enzyme present in the reaction mixture. Since these reaction mixtures contain about 1 nmol of enzyme and as much as 50 nmol of nucleotide, it should be clear that this type of reaction would be just barely detectable (if at all) if the standard approach of acidifying the entire reaction mixture were employed. The other type of interaction of nucleotide with the enzyme is several orders of magnitude faster. This is the hydrolytic activity that is the subject of the present communication and which can be detected by standard enzymological procedures. Complexing of ATP with a hydrolytic site on the enzyme would give rise to what we would call a "loose" complex—*i.e.*, one which forms and dissociates much too quickly to be detected by exclusion chromatography on Sephadex G-50M columns. It should be of great interest to determine whether there are really two different classes of sites on the enzyme, "tight sites" and "loose sites," or whether there is just one basic class of sites which can alternately exhibit two different modes of behavior.

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Control of Conformation of α-Chymotrypsin through Chemical Modification†

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ABSTRACT: Oxidation of Met-192 of α-chymotrypsin enhances the stability of the active substate of this protein relative to the inactive substate and also reduces the autolysis rate making this derivative the currently most favorable one for studies of mechanism. A new reagent, trichloromethanesulfonyl chloride, oxidizes Met-192 to the sulfoxide form in a rapid stoichiometric reaction. In the usual pH range employed for chymotrypsin kinetics this derivatized enzyme exists entirely in the active substate until at least pH 9.1. In the course of this work we found that *N*-bromosuccinimide and *N*-chlorosuccinimide oxidize Met-192 at least as rapidly as they oxidize any indole groups. It is probable that this heretofore unrecognized behavior is common in studies with these reagents. The further oxidation by H_2O_2 in urea of Met-180 produces a dimethionine sulfoxide derivative which has a rapid autolysis rate especially at slightly higher temperature. Under conditions at which autolysis is minimized the derivative has relatively high amidase activity in contrast to previous reports.

There are a number of conformational substates which characterize α-chymotrypsin in solution (Hess, 1971; Lumry and Biltonen, 1969); any experimental procedures which can either limit the number of substates or explain their importance to the catalytic process will aid significantly in our understanding of the mechanism of action of this enzyme. We have used chemical modification to achieve these ends. It is not yet generally realized that the "inactive" species forms a significant fraction of the total enzyme in α-chymotrypsin solutions at all pH values usually chosen for investigation (Fersht and Requena, 1971; Fersht, 1972). The equilibrium between active and inactive forms is a sensitive function of substrate concentration, pH, and temperature, so that in effect a major fraction of previous quantitative studies of the enzyme with or without small specific molecules is in error. In this paper we show that a well-known chemical modification of the enzyme eliminates the inactive species over a wide pH range without qualitative change in catalytic behavior. Although additional substates exist (Lumry and Biltonen, 1969), the derivative provides considerable improvement in substate control leading to a respectable beginning of quantitative studies of this enzyme.

Previous work involving chemical modification of chymotrypsin is extensive (Dixon and Schacter, 1964; Hess, 1971; Kosman and Piette, 1972; Nakagawa and Bender, 1970; Schacter and Dixon, 1964). Reagents have been developed which are specific for either active-site residues or for the amino acids which are believed to contribute significantly to maintaining this enzyme's tertiary structure. We emphasize that it is crucial to all chemical modification experiments that such reactions be "clean" and well defined. Not only must it be clear which residues have been modified and how, in addition interpretation of resulting changes in the enzyme's activity in terms of "specificity," "structure," "conformation," etc., must be made with the utmost caution (Dixon and Schacter, 1964). Changes in enzymatic properties which are ascribed to chemical modifications of the active site can in fact be due to conformational changes which render the enzyme inactive (Dixon and Schacter, 1964).

In this paper we reexamine a number of well-known α-

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